Original Research Article

Synthesis and Biological activities of Cu-Nanoparticles from *Aspergillus niger*

ABSTRACT

Nanotechnology has received tremendous attention because its applications have expanded in a variety of fields. The biological route for synthesis of nanoparticles become more demanding as it is eco-friendly, low cost and not time taking procedure. In this research *Aspergillus niger* filtrate used as reducing agent to biosynthesized copper nanoparticles (CuNPs) under controlled parameters i.e., pH, temperature and time. Synthesized CuNPs were confirmed by UV- Visible spectroscopy and further characterized by scanning transmission electron microscopy, Fourier transform infrared spectroscopy. The UV- Visible spectroscopy exhibit maximum peak 540nm which confirmed the formation of CuNPs. FTIR shown two maximum peaks 3339cm^{-1} and 1638 cm^{-1}. These peaks clearly represent presence of O-H stretching and –C=C- stretching respectively. The size ranges of CuNPs between 100nm-500nm with spherical shapes. The anti-microbial activity was tested against gram positive and gram-negative bacteria and showed significant antibacterial potential of CuNPs. Radical scavenging activity was confirmed by DPPH assay. The results of antioxidant activity indicated IC50 value of CuNPs was 59.10ug/ml. Thus, CuNPs synthesized through biological route could act as good antibacterial as well as antioxidant agents.

Keywords: *Aspergillus niger,* nanoparticles, copper, antibacterial, antioxidant

INTRODUCTION

Nanotechnology is one of field of science where we study about small particles having sizes approximately 0.1 to 100 nanometres, measured at nanoscale. Nanotechnology is able to synthesize particles at nanoscale level. Present-day nanotechnology continuously gain attention because of their potential to produced nanoparticles and their use in drug delivery system as nanomedicine. This technology exactly tackle human diseases before time [1a]. Advanced developments in science leads toward progress very fast to the exact dimension in

production of nanoparticles. Nanoparticles have been extensively used to deliver to specific target areas and to maintain stability against enzyme degradation . Being produced by-product using nanotechnologies in science disciplines is known as nanobiotechnology. The Biotechnology advancements have created limitless possibilities for genetic diagnostics and clinical therapy [2]. Due to the exclusive properties of nanomaterials like antimicrobial, anticancer, catalytic activity, and optical properties, these metal nanoparticles (NPs) have been explored extensively [3]. Drug designing at nano-scale level need detailed research because of its potential benefits, including solubility, drug release profiles, diffusivity, bioavailability, and immunogenicity, that is why it becomes most advanced technology in the field of nanoparticle applications. All phases of clinical treatment the nanoparticles have been proven to be effective in several new tests to treat and diagnose illnesses.

The use of metal-based nanoparticles in diagnosis and treatment is an area of study that led to more widespread use of nanomedicines in the future. As, most stabilizing nature of copper, biosynthesis of CuNPs offers higher preferences in comparison with others metallic nano-particle. Their high conductivity characterizing exist naturally. They may have emerged as attractive candidates for numerous medicinal applications due to their characteristics. There are some major applications of copper based nano-drug including targeted drug-transporting and gene-therapy, molecular tracking & detection all clinical diseases like cancer, diabetes, and atherosclerosis [4]. The advancement of less hazardous technologies for producing metallic nanoparticles is critical. A very reliable approach to Exploiting nature's diverse biological resources to achieve this goal [5]. There are variety of system for the synthesis of Nano-particles. These two most broadly used methods are: Bottom-up procedure and Top-down procedure. These approaches are further considered as a sub-classes which totally based on response conditions. Some steps used in synthesis of nanoparticles comprising chemicals, mechanical and biological method. Chemical method include plasma, Micro emulsions, sonochemical reduction, microwave irradiation, ultrasound irradiation, Pulsed laser method and laser. These all are chemically based methods which are widely used for the synthesizing of nanoparticles. It is because large numbers of nanoparticles may be produced in a short amount of time with presize shapes and sizes. It is very not eco-friendly method [6]. Mechanical procedure are same as physical method for the synthesis of CuNPs. Physical processes, are mechanical milling, induced heating & spraying which is not harmful, although they are difficult and time-consuming. All these methods are basically produced toxic products which cause health problems sometimes. These methods are still gained its

status but very complexed. However, the biological methods are more suitable for production of nanoparticles. As biological route does not require expensive and harmful substance. It is very extensively used just due to low prices, greater efficiency, environmentally friendly, and easy to handle in labs. In all plants and other organisms (e.g. cyanobacteria's) not need stabilizing agent as enzymes are available in their bodies which immediately eliminate toxicity chemical whenever observed. Biological methods are suitable for producing therapeutically-based nanoparticles. Using biological method for generating nanoparticles includes involvement of different species e.g. various plants, different bacterial strains, different type of fungi, and algae. These species are utilized to produced nanomaterial through biological means are effectively for diseases problems and side effects. Biosynthesis of NPs bought interest in all science fields with act as cure against microbial plant diseases like whitefly muted cotton and *Medicago sativa* [7]. Metallic nanoparticles have considerable attention in contrast to all other metallic nanoparticles due to their surface to volume ratio, small size, resistance to oxidation. CuNPs extensively used instead of expensive noble metal and performed diverse functions for instance transfer of heat and in inject printing. Nanoparticles produced from copper gain more attraction then nanoparticles produced from other metals because copper is an important micronutrient for a variety of biochemical mechanisms in both health and disorder [8]. CuNPs that might been employed in variety of science fields [9]. CuNPs have a lot of potential for treating microbial diseases since they have antimicrobial properties against both gram negative and gram positive bacteria [10]. As, most stabilizing nature of copper, biosynthesis of CuNPs offers higher preferences in comparison with others metallic nano-particle. Their high conductivity characterizing exist naturally. There are some major applications of copper based nano-drug including targeted drug-transporting and gene-therapy, molecular tracking and detection all clinical diseases like cancer, diabetes, and atherosclerosis [4].

MATERIALS AND METHODS

Collection of Fungus

Spoiled mangoes were collected from the mango farm. Mango is big source of *Aspergillus niger* [11].

Chemical Collection

20g of sabouraud dextrose agar (SDB) and potatoes dextrose agar (PDB) was obtained from chemical store of Institute of Molecular Biology and Biotechnology. The molecular mass of the copper sulphate salt is 159.6g/mol. All chemicals e.g., DMSO, DPPH, ethanol of analytical grade were purchased from Sigma Aldrich.

Isolation and Purification of Filamentous Fungus

Separated green spots of fungus from mango fruits with the help of sterilized wire-loop. These all mango fungal-spores were then cultured in patri-plates having fungal growth media (SDA) sabouraud dextrose agar. The fungal-growth (SDA) media (25.12g) was prepared in 500ml flask having 400ml of distilled water, then autoclaved. Sterilized media was poured in sterilized glass patri plates (Jain et al., 2011). For isolation, SDA containing media plates were prepared then fungal spores inoculate on solidified media with sterilized wire loop. After this step, plates were placed in the incubator for 2-3 days at 37°C temperature for fungal growth [12]. The individual fungus-spores picked for further purification. Sub-cultured 2 to 3 times to obtained pure fungus culture. After purification, stored all plates having purified fungus put at 4 °C. The purified fungus-spores maintained at 28°C with pH 6.5 for longer use. The purified culture used for screening of strains [13].

Identification of Purified Fungus

Purified fungus was identified by following methods:

Morphological Identification

Morphological identification of purified fungus was done after 4 days by observing fungus shape, colonies growth, colour, appearance, fungal-diameter and blackish velvety spores. These all are macroscopic characteristics of fungus [14].

Microscopic Identification

Microscopic identification of fungus done after 5 days of incubation. Although, after preparing the slides, microscopy was done. Spores of purified fungus was separated with the help needle. Placed on a slide and teased until large structures were shattered completely. Put a drop of water on slide then placed a coverslip. Tissue paper used to remove extra water. On the slide, mentioned a name to be used later. Then, set slide on microscope perfectly. The slide under microscope at 40 X magnification to observed characteristics of fungus [15,16].

Molecular Identification

The first step of molecular identification is DNA extraction. Fungal DNA was extracted through Cetyl Tri-methyl Ammonium Bromide (CTAB) technique along some modifications[17,18]. Fungal hyphae (0.5g) was shown fast growth in SDB, which extracted by filtration through whattmann filter paper no.1 and dried it then freeze it. Freeze dried fungal spores used for filtration process. For 25ml Cetyl Tri-methyl Ammonium Bromide buffer prepared in 200 ml distilled water, after mixing of 10 ml EDTA, 25 ml Tris-base and 20g NaCl for 25 minutes. CTAB extraction buffer used to properly grinding of fungal conidia's structures and noted that cell-walls of fungal structures broken completely, this grinding was done through the help of pestle and mortar. Add little more CTAB buffer if required. After powering homogenized mixture was shifted to 1.5 ml all tubes kept in water bath for 25 minutes at 65℃. Then tubes left for 30 minutes at room temperature. Add chloroform and isoamyl alcohol (24:1) solution in appendrof tube as equal volume to reaction mixture. Ependroffs tubes were inverted after every 10 minutes. Then centrifuge for 10 minutes at 9000 rpm. After that, this solution was removed and supernatant contained DNA are transfer into another tube and chilled added with same volume. Tubes were again centrifuged at 9,000 rpm for 10 minutes. Pellets of DNA washed with ethanol after centrifuge. Centrifuged it for 5 minutes at 9000 rpm. Through inverting method, tubes inverting every 3-5 minutes. Upper impurities removed 3 times. The tubes had pure DNA pellets. All pured pellets of fungal- DNA stored at -20 ℃ for further use [18]. The UV-spectrophotometer used for fungal DNA quality. After that dilute the samples. Diluted DNA sample added into 99 well plate and samples quantified by measured absorbance of fungal-DNA at A_{260}/A_{280} nm. Blank is distilled water. For amplification of isolates at molecularly by PCR Amplification. 18S ribosomal DNA was done through this amplication. The main components needed in PCR amplication. The reaction mixture was prepared using 4µl of pured DNA sample, nuclease, 12.5 µl of 2X Master-Mix contained taq-DNA polymerase, buffer, dye, dNTPs 0.4 µM, reverse and forward primers required and 2-3 drops required to spread on reaction mixture. Bio-Rad thermal cycler present in Laboratory of IMBB used for PCR amplification. Initial-denaturation of Fungal isolate of rDNA which carried out at 95℃ for 6 minutes the 35 cycles required for denaturation at 95℃ for 35 sec, primers annealing need for 35 sec at 60℃, last extension done in 10 min at 72℃. After this, one % Agarose-gel required for PCR product analysis.

Sequence Analysis

Similarly, the PCR reaction was conducted by using 30 µl of master mixture activation of *Taq* polymerase for 3 minutes at 94℃, 35 cycles of denaturation at 95℃, annealing at 55℃ and extension of primer at 72℃. For purification of PCR amplified product, known as 96 well plate was used. Terminator-cycle Sequencing Kit used to perform sequencing of fungus SN16. Samples having amplified DNA products suspended into Hi-Di for-Amide, incubated for 6 minutes at 95℃ for heat-shock treatment and kept in Ice for 5-6 minutes. The analysis of sequences DNA analyzer used carefully [19].

Phylogenetic Tree

The phylogenetic-tree having capabilities to detect Evolutionary Relationships among organisms of same species because of sequence similarities in genetic history. Eventually, Clustal Omega tool used to identify pair-wise genetic distances and beside sequences joining used to build phylogenetic tree. Clustal Omega also important to achieve unweight rDNA results easily. It is presented in Dendtrograms forms. The Alignment of rDNA of fungus stain (SN16), using ‘’Basic Local Alignment Search Tool’’ BLAST at NCBI used to detect the correlation-ship of sequences of fungus *Aspergillus niger* [20,21]. The Omega tool was used for construction of phylogenetic tree.

Biological Synthesis of Cu-NPs

Preparation of Biomass Mat

The two types of culture media i.e., SDB and PDB prepared in 250ml flask. 12g of PDB and 12g of SDB powder added in 150ml of distilled and autoclaved water then mixed through spatula/stirrer. This prepared media of SDB and PDB autoclaved. This autoclaved media then cooled properly. After cool-down step the purified fungus *Aspergillus niger* spores inoculate in both SDB and PDB media in Laminar Flow-Hood (LAF) cabin. These medial flasks kept in rotatory shaker for 7-10 days, 120 rpm speed and at 28°C. After incubation, flasks containing fungal biomass removed from the shaker [22].

Preparation of Fungal-Filtrate

After incubation/inoculum, biomass mat harvested by using Whatman filter paper No 1. All impurities present in biomass was removed by washing three time with water. The mat after 3 washing weighed and 9.8g of biomass again poured into the washed flask containing 200ml deionized water and kept in arbitrary shaker at 120 rpm for 1 day incubation. After that,

biomass was filtered 1st by using Whatman filter paper No.1 and then with syringe filter of 0.2µm. The PBD filtrate and SDB filterate obtained used for Cu-NPs synthesis [23].

Synthesis of CuNPs

Different dilutions of Cu-salt ($CuSO_4$) i.e., 10, 15, 20mM mixed with the fungal-filtrate with the ratio of 1:1 at room temperature. On addition of $CuSO_4$ solution, a colour change was detected by naked eye. The synthesized nanoparticles placed in arbitry shaker for one day incubation at 37°C

Drying of CuNPs

Followed the protocol for drying copper nanoparticle. After one day of incubation CuNPs were centrifuge at 1200 rpm for 5 minutes. This process repeated for 3 times. The upper watery extract discard and washed CuNPs with distilled water. These steps performed thrice and after 3 washing the only NPs were left in the form of pellets. These pellets were poured in the Aluminium foil. The pellets heated at 100 °C for one day. The next day pellets removed from hot oven. Through this, pure dried form of nanoparticles obtained [24].

Characterization of Dried CuNPs

CuNPs characterized by different techniques including UV-spectrophotometric, scanning transmission electron microscopy and Fourier transform infrared analysis etc.

UV-Spectrophotometer

The CuNPs characterized by UV-visible spectrophotometer. CuNPs absorbance was measured at 1 nm resolution by UV-visible spectro-photometer after 1 day incubation, one ml of (Cu-NPs) taken at different concentration with wavelength range was adjusted at 200-800nm [25]. For UV-spectrum Micro-plate spectrophotometry (96 well-plate) was used for detection of CuNPs. The distilled water used as blank. Sample data was loaded sequence wise after added blank. Recorded all sample data and data graph plotted on excel sheet.

Scanning Transmission Electron Microscopy (STEM)

Scanning Transmission Electron Microscopy (STEM) used to analysis the sizes and shape of CuNPs. The scanning instrument (resolution 5.25 A^o) used electron light beam to produced image on screen. This provides exact size of CuNPs in high resolution. It can detect even

1nm small sized particle. Scanning electron micrograph was taken from instrument of JSM-6380 SEM model. Before experiment, samples were filtered and dried [22].

Fourier-Transform Infrared (FTIR)

FTIR spectrometer used to determine functional groups that bind with CuNPs. FTIR analysis was performed with the help of interferometer. CuNPs diluted in potassium bromide at 1:100. One drop of solution placed on sample holder. Spectrum noted in the series of 1000-3500cm^{-1} wavelength [26].

Antibacterial Activity of CuNPs

For antibacterial procedure, the microbial cultures obtained from laboratory of Institute of Molecular biology and Biotechnology. Antibacterial activity was performed against gram negative bacteria's *Escherichia coli, Klebsiella pneumoniae, Salmonella enterica* and gram-positive bacteria's *Bacillus subtilis, Micrococcus luteus* and *Staphylococcus aureus.* For anti-bacterial activity, agar well method performed. Media plates containing nutrient agar were prepare by adding 4.2g nutrient agar in 150ml of distilled water and kept it in autoclave machine for 90 mins. Cooled down media completely then poured into glass petri-plates in LAF hood. Prepared media was not touched until solidified, after the wells were prepared by steel borer (6mm). Sterilized swabs were used for microbial culturing on plate. Swabs rolled around the wells perfectly. CuNPs dilutions 10mM, 15mM and 20mM were added into wells using tips and pipette 10-100ml. NPs (100ul) add into wells. The standard was drug (Ciprofloxacin) added in all plates and these plates were left for 5 mins to allow to diffuse properly. All plates were kept in the incubator for one day incubation at 37 °C. After one day, remove plates from incubator and measured zone of inhibitions (ZOIs) using scale and maker. Experiment was repeated 3 times [27, 28].

Antioxidant Activity of CuNPs

DPPH scavenging activity was performed to evaluate antioxidant potential of CuNPs. Prepared NPs samples dilutions at different concentrations (10-100µg/ml) then made dilution of ascorbic acid similar to samples. The reaction was observed after DPPH changes its colour from purple to fade yellow in methanol solution. After fully vertexes, the reaction mixture was kept at RT for 30 minutes in the dark. The colour shades changed to fade yellow colour in the presence of antioxidants. The colour change observed which showed that breakdown of oxygen. Antioxidants' ability to donate hydrogen when DPPH to be seems. The 99 welled

plate used to spectrophoto meter analysis. Graph should in proper trend and straight line. At 517 nm, the mixture's absorbance was determined spectrophotometrically [29]

%DPPH Radical-Scavenging Activity = {(control absorbance – sample absorbance)/control absorbance} ×100

Inhibition graph curve prepared after IC 50 value calculated on excel sheet.

RESULTS

Identification of Isolated Fungus

After screening, the purified fungus obtained after 5-7 days. The dark coloured purified fungus obtained. The purified fungus contained single colony and black velvety spores spread all over colonies clearly shown below in Figure 1. The fungal-isolate change its colour behaviour from whitish to deep brownish-black colour and straw pale pattern of colonies from backside. The growth of fungus with globular shaped shown in Figure 1.

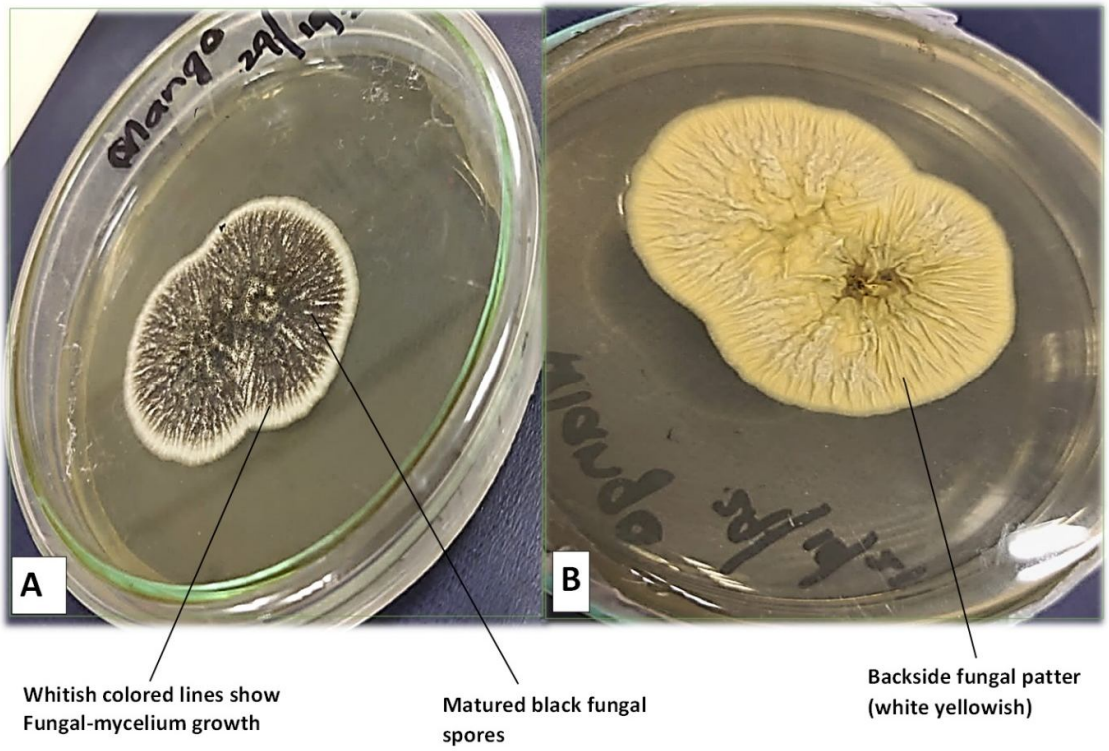

Figure 1 Greyish black velvety textured *Aspergillus niger*

The microscopic characterization showed hyphae without septa, globular shaped matured conidia had dispersed conidiophores. Microscopy identification also showed that produced spores were between 200-250nm and spores shape were spinose. The stipes was white in colour around the apex. The flask shaped phialides was completely covered the vesicles. Long thin tubular hyphae were unbranched (Figure 2).

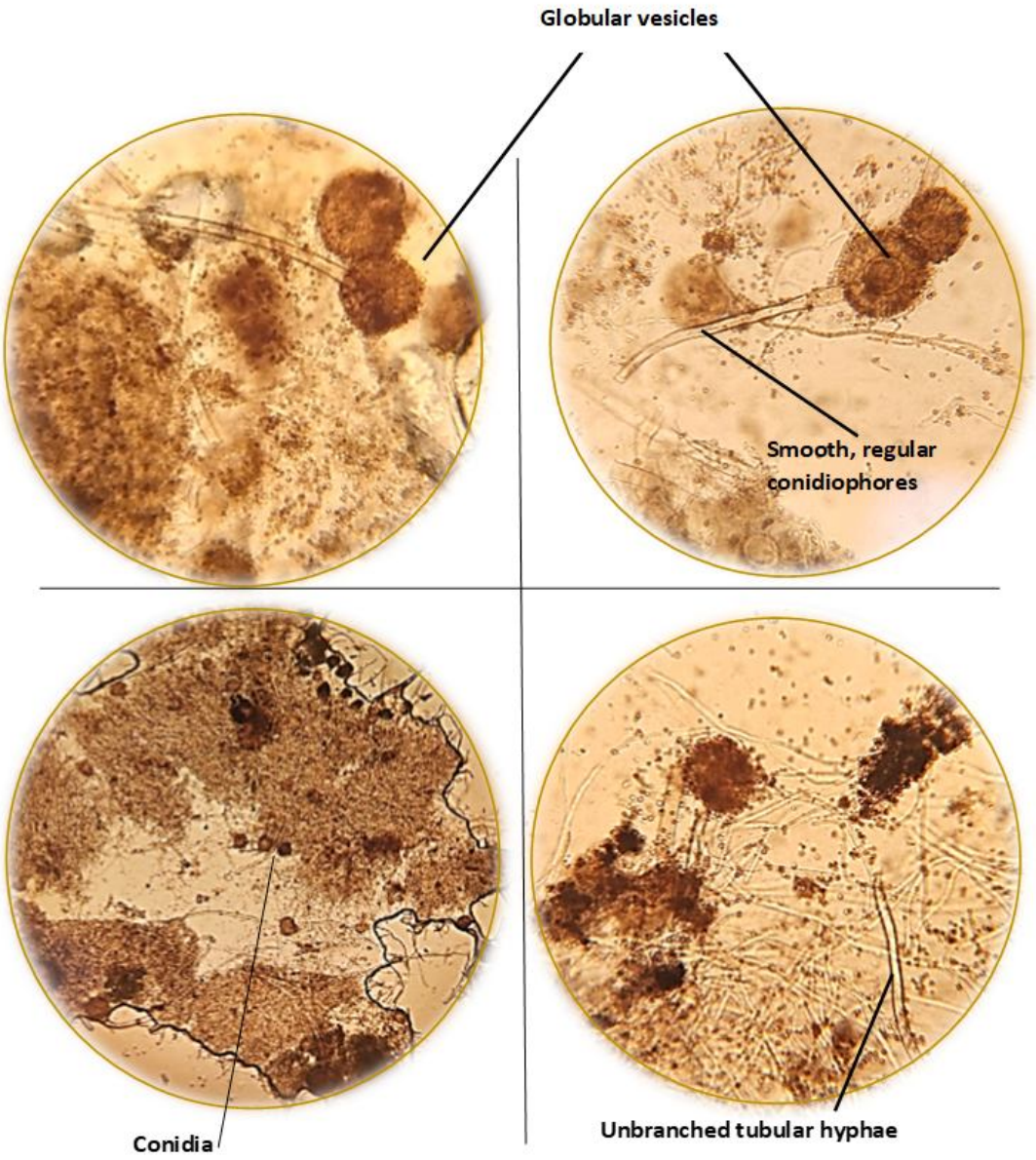

Figure 2 Microscopic evaluation showing detail structure of fungus, contained long unbranched hyphae, globular conidiophores

Molecular Characterization

The fungus-isolate was characterized by sequence analysis with 18S-rDNA region. The molecular analysis clearly shown that purified fungus strain was *Aspergillus niger*. It was proved by NCBI website. NCBI-server given BLAST results. The confirmation of fungal-strain sequences which was resemble with *Aspergillus niger* sequences present in the NCBI database.

Phylogenetic tree was construct through online website, Clustal Omega by using option MUSCLE. Then sequence was submitted to NCBI with their allotted number. The phylogenetic overview shown in Figure 3.

Phylogenetic tree

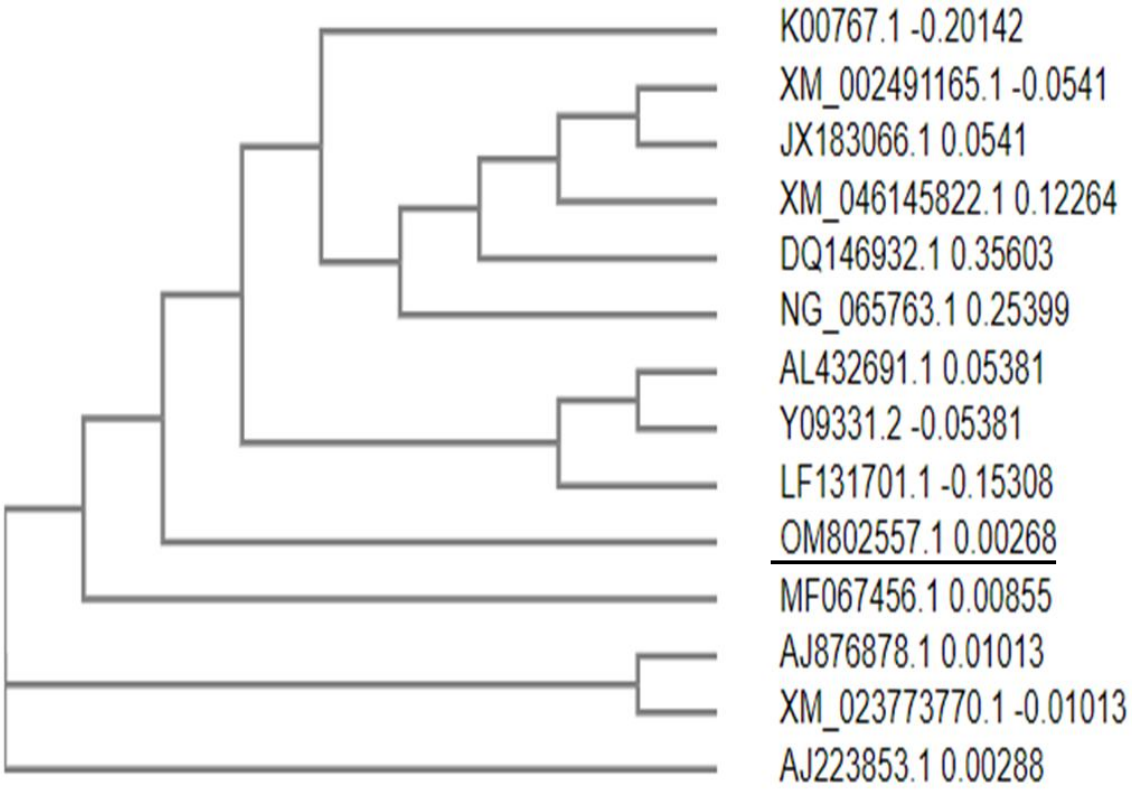

Figure 3 ____ *Aspergillus niger* strain with 0M80255.1 allotted number, the partial sequences of small subunit of gene rRNA

The phylogenetic tree representing evolutionary linkage of *Aspergillus niger* strain SN16.clustral omega used for designing tree. Maximum likelihood process used to compute distance and units by number-based substitution per site.

Biologically Synthesized CuNPs

The biosynthesis of CuNPs by mixing *Aspergillus niger* filtrate with $CuSO_4$ salt. The filtrate was cell-free so used as reducing system in CuNPs production. Filtrate of *Aspergillus niger* reduced the copper-salt and it proved by change in colour from pale white to light blue. The formation clearly shown in Figure 4. Controlled parameters e.g., pH 7 and reaction temperature 37 ^{o}C. 1:1 ratio (filtrate: salt) were used.

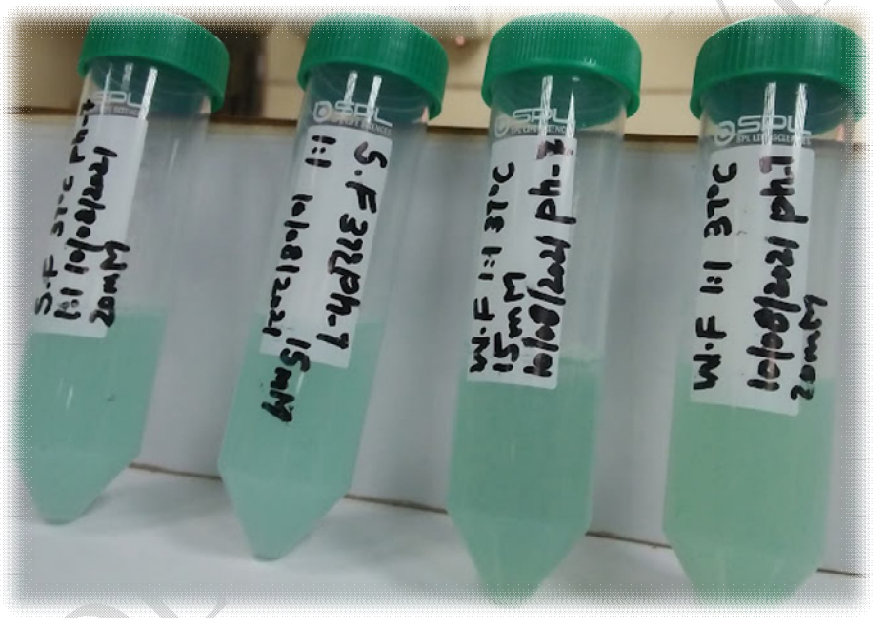

Figure 4 Change in colour clearly supported the copper-nanoparticles formation

Characterization of Biosynthesized CuNPs

UV-Visible Spectrum Analysis

The UV-spectrum was carried out from 200-800 range of wavelength which helped to confirm the synthesis of copper-nanoparticles from *Aspergillus niger*. Similarly, the broad-spectrum range was the 350-550nm, this range supported maximum absorbance spectrum. It

was observed CuNPs showed maximum absorption peak at 540nm. It was clearly shown in graph that filterate of fungus have zero absorption-peak between 220-600nm wavelength. The copper nanoparticles showed maximum excitation at different points by absorbance. The CuNPs indicate maximum peaks at different points because of surface-plasmon resonance spectrum by this reduction of $CuSO_4$ into NPs. The peaks are shown below in (Figure 5).

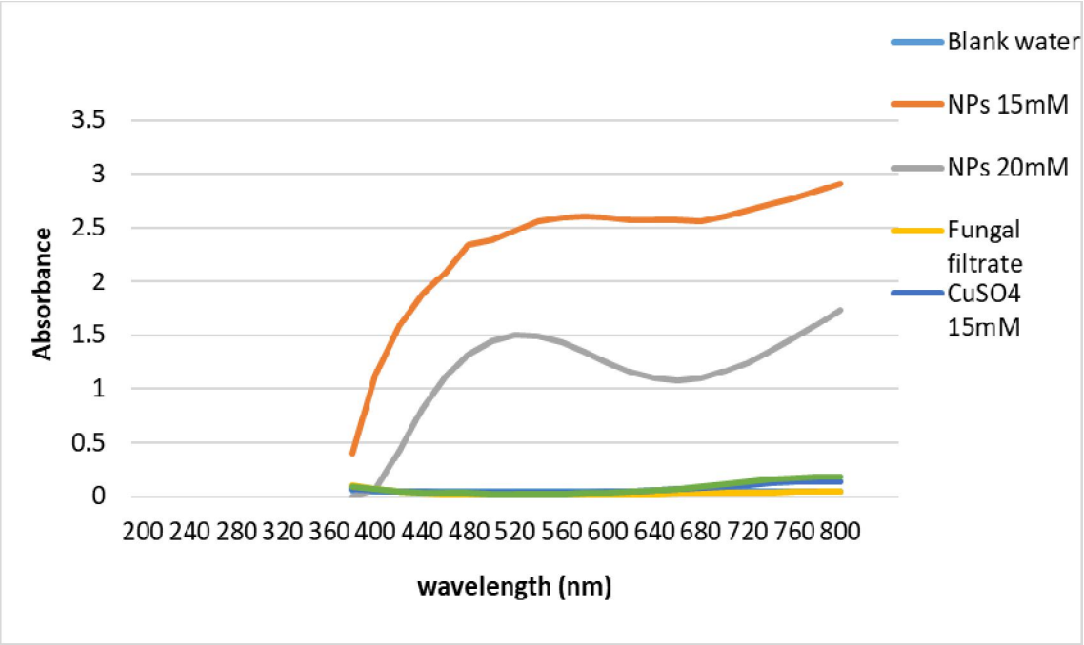

Figure 5 Graph curve showed the absorbance of NPs at different wavelength

Fourier Transform Infrared Spectroscopy (FTIR)

Biosynthesized copper nanoparticles absorbance peaks were observed by FTIR apparatus. The peaks were obtained which analyse the stabilized CuNPs and high peak observed at 3330.07cm-^{1} by this, phenolic ions bind with O-H functional group express stretching of O-H bond. The wave region of FTIR spectroscopy was at 1000-3500cm^{-1}. The -C=C- bond stretching which supported the presence of (alkene) functional-group in CuNPs at high absorption peak 1636.91cm^{-1}. The results shown in Figure 6. These peaks identify the functional bonds and furthermore, involved in capping and binding of CuNPs.

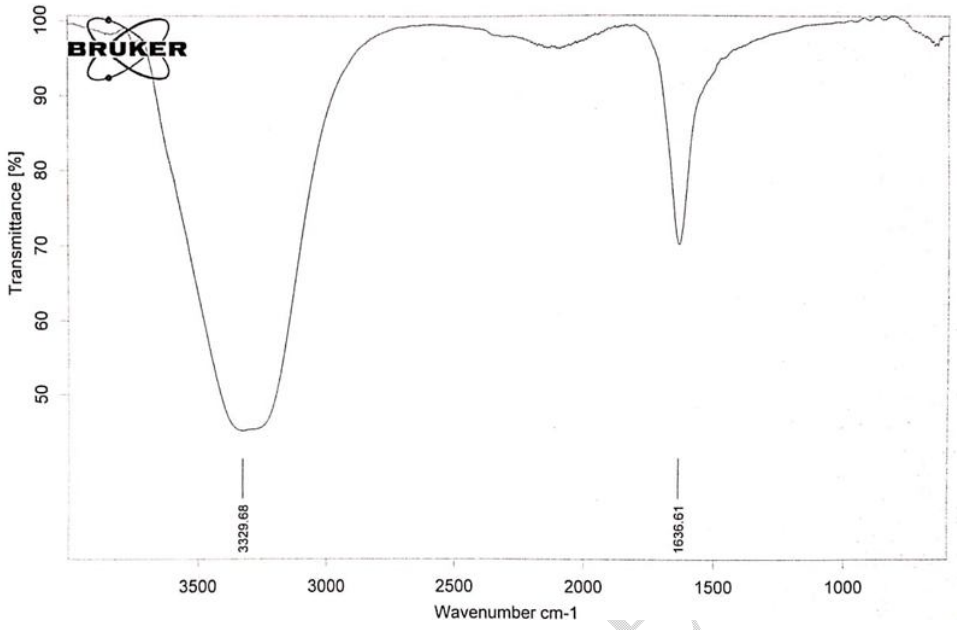

Figure 6 CuNPs analysis by FTIR spectrum

Scanning Transmission Electron Microscopy (STEM)

The STEM images provided information about elemental composition of a single atom. Same principal as SEM and TEM. STEM is fast mode of analysis. This microscopy supported the imaging of secondary electrons. Its importance is that multiple operations of characterization done simultaneously. Its gives actual atomic bonds present inside nanoparticles. STEM characterization supported 0.2nm resolution at 200kV accelerating voltage. In this analysis not only size and shape were observed but size distribution, NPs also counted. Different magnification compute results 25000X-200,000X nm range. The STEM micrograph showed the size of CuNPs range between 50nm-125nm while exhibit spherical shape (Figure 7).

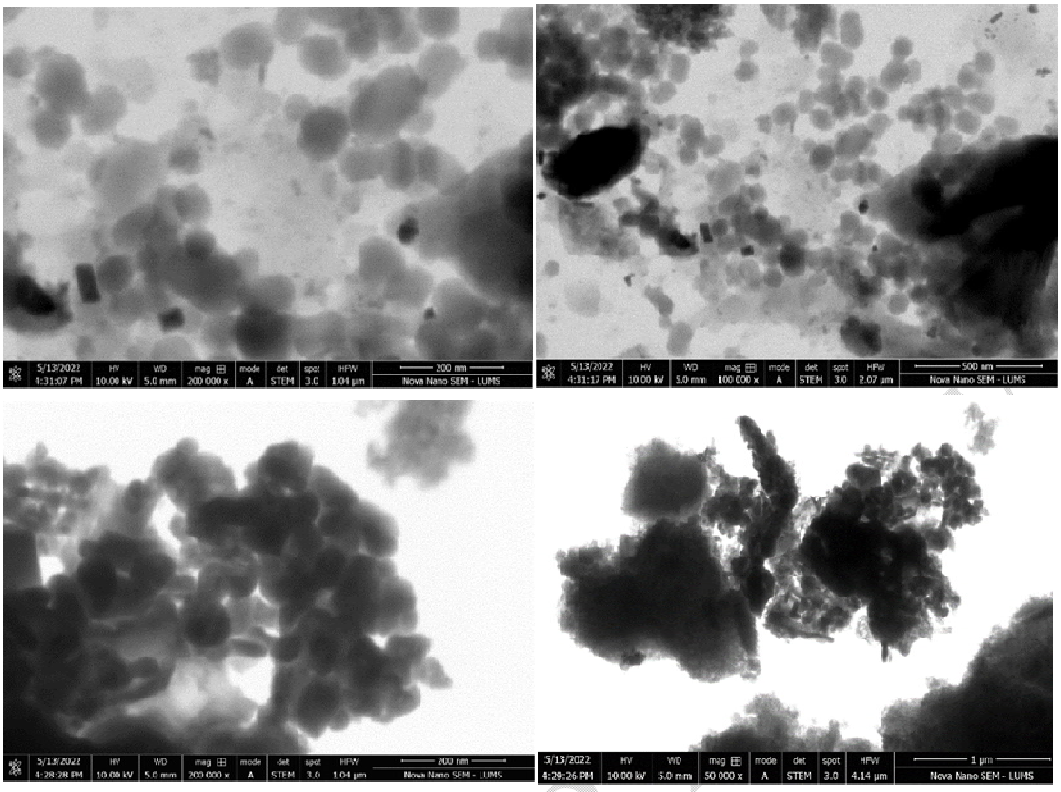

Figure 7 STEM analysis of CuNPs

Biological Activities

Antimicrobial Activity of CuNPs

Anti-bacterial activity of fungal-based copper-NPs analysed against 6 bacteria including: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis* and *Salmonella enterica*. The antibacterial activity of Copper-NPs synthesized from 15mM salt concentration was observed to be 27mm, 15mm 23mm, 14mm, 13mm and 11mm respectively. Copper nanoparticles showed significant antibacterial efficacy results against bacteria (Figure 8, Table 1).

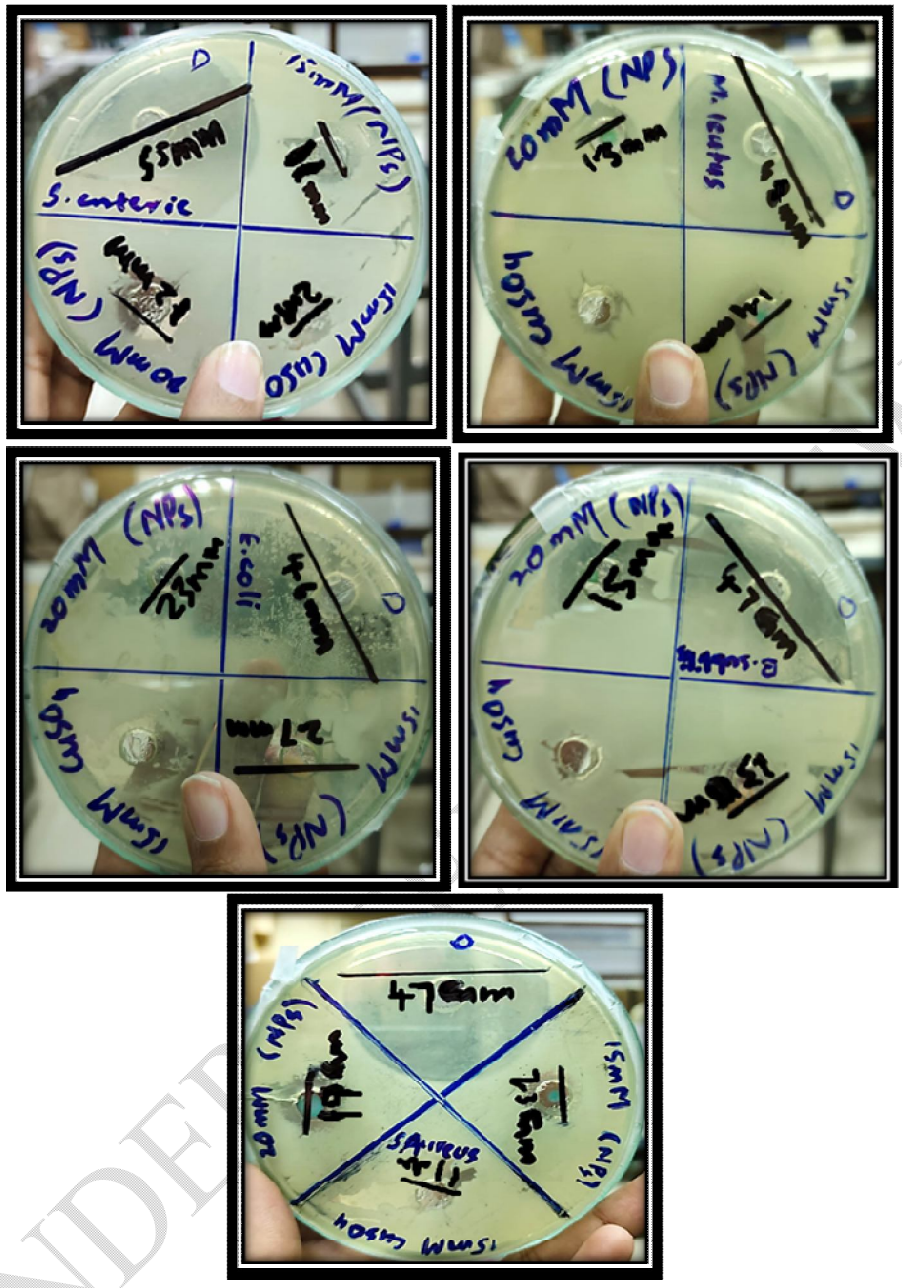

Figure 8 Antibacterial activity of biosynthesized CuNPs

Table 1 CuNPs activity against different bacterial strains

MICROBIAL STRAINS	ZOI of DRUG (Ciprofloxacin)	ZOI of CuNPs 15mM NPs	ZOI of CuNPs 20mM NPs
Escherichia coli	46mm	27mm	23mm
Pseudomonas aeruginosa	55mm	15mm	16mm
Staphylococcus aureus	47mm	23mm	19mm
Micrococcus luteus	48mm	14mm	13mm
Bacillus subtilis	47mm	13mm	15mm
Salmonella enterica	55mm	11mm	12mm

Antioxidant Activity of CuNPs

Antioxidant activity of nanoparticles and standard (ascorbic acid) was evaluated at varying concentration (10-100µg/ml). The IC50 value was calculated, and results showed that IC50 value of CuNPs was 59.10µg/ml as shown in Table 2.

Table 2 Antioxidant activity of Standard and CuNPs

Concentration	% Inhibition of Standard	IC50 (µg/ml)	% Inhibition of CuNPs	IC50 (µg/ml)
10	44.05	**38.134**	37.06	**59.10**
20	46.85		39.86	
40	51.04		44.40	
60	52.44		50.34	
80	57.69		54.89	
100	65.73		61.53	

DISCUSSION

The aim of this work was to develop a microbial-based method for the synthesis of nanoparticles. Fungi are attractive candidates for the biogenic production of copper nanoparticles because they have high metal resistance and are easily treated. The first metal used to make the NPs was copper. For the production of copper nanoparticles, *A. niger* was used. *Aspergillus* and several other fungal species have been used for the manufacture of nanoparticles, according to several researches. In order to quantify the nanoparticles in the 250–600 nm wavelength region, which indicates the stability of NPs, UV–visible spectral analysis was performed. It was found that there was a little variation in the absorption peaks for various parameters. For the synthesis of NPs, the following precursors are involved and numerous physical and chemical methods are being employed for the synthesis of CuNPs, however owing to constraints, development of more acceptable and appropriate procedures and techniques that are eco-friendly, cost efficient, and readily scale-able is a crucial demand. Because of its simplicity, applicability as one step process, and production at scalable parameters, biological synthesis has proven to be more important than physical and chemical methods. CuNPs micro-synthesis by *Aspergillus niger* is a highly effective approach. Because copper ions must be reduced and copper nanoparticles must be stabilised, an external reducing and capping agent is required. Furthermore, there are no hazardous chemicals involved, making this procedure ideal. CuNPs characterized by UV-spectrophoto-

meter.it was also evaluated by other important methods of characterization are FTIR, STEM. The spectro results revealed that copper Nanoparticals was synthesized using biological method, by observing peak at 540nm.the reported peak for CuNPs is 480nm so confirmation of synthesis of nanoparticle by metallic salt. The FTIR results showed the presence of Functional group in NPs. There was two peaks observed one peak shown presence of OH group and other one show presence of phenolic group which tightly binds to OH group. Antibiotics such as cephalosporin, tetracycline, and streptomycin are currently available. These antibiotics have limitations because bacteria have developed resistance to them. The impact of drug resistance on human health is significant. Antibiotic resistance has risen dramatically in recent years as a result of usage. Copper nanoparticles have been identified as a possible antibacterial agent for example *E.coli, staphylococcus aureus, Micrococcus luteus, Bacillus subtilis* by observing and measuring zone of inhibition with scale. This research shown the significant results of Cu-NPs. The tested strains were 27mm, 15mm, 14mm and 13mm respectively. It indicates the mechanisms of bacterial action of nano-particle to damage the membrane extend of inhibition depends on the concentration of nanoparticles as well as initial bacterial concentration. The reason for this could be that smaller particles absorb more tightly on the surface of bacterial cells, disrupting the membrane and allowing intracellular components to leak out, killing the bacteria.

CONCLUSION

The CuNPs synthesized from biological route by using cell free filtrate of *Aspergillus niger* as reducing agent. Myco-synthesis of nanoparticles seems eco-friendly and coast effective method. CuNPs characterized through FTIR, and STEM showed functional groups attached to nanoparticles along with size and shape. Furthermore, myco-based CuNPs showed significant antibacterial action against different pathogenic bacterial strains and antioxidant potential with IC50 value of 59.10µg/ml. Further studies should be done to explore the other biological applications of CuNPs.

REFERENCES

1. NOOR, S., SHAH, Z., JAVED, A., ALI, A., HUSSAIN, S. B., ZAFAR, S., ALI, H. & MUHAMMAD, S. A. 2020a. A fungal based synthesis method for copper nanoparticles with the determination of anticancer, antidiabetic and antibacterial activities. *J Microbiol Methods,* 174**,** 105966.
2. PRAETORIUS, N. P. & MANDAL, T. K. 2007. Engineered nanoparticles in cancer therapy. *Recent patents on drug delivery & formulation,* 1**,** 37-51.
3. GUPTA, C., RABANI, M. S., GUPTA, M. K., TRIPATHI, S. & PATHAK, A. 2022. Nanoscience in Biotechnology. *Diverse Applications of Nanotechnology in the Biological Sciences.* Apple Academic Press.
4. MODY, V. V., SIWALE, R., SINGH, A. & MODY, H. R. 2010. Introduction to metallic nanoparticles. *Journal of Pharmacy and Bioallied Sciences,* 2**,** 282.
5. THAKKAR, K. N., MHATRE, S. S. & PARIKH, R. Y. 2010. Biological synthesis of metallic nanoparticles. *Nanomedicine: nanotechnology, biology and medicine,* 6**,** 257-262.
6. HORIKOSHI, S. & SERPONE, N. 2013. Introduction to nanoparticles. *Microwaves in nanoparticle synthesis: fundamentals and applications***,** 1-24.
7. KUPPUSAMY, P., YUSOFF, M. M., MANIAM, G. P. & GOVINDAN, N. 2016. Biosynthesis of metallic nanoparticles using plant derivatives and their new avenues in pharmacological applications–An updated report. *Saudi Pharmaceutical Journal,* 24**,** 473-484.
8. ANGELOVA, M., ASENOVA, S., NEDKOVA, V. & KOLEVA-KOLAROVA, R. 2011. Copper in the human organism. *Trakia journal of sciences,* 9**,** 88-98.
9. AMALIYAH, S., PANGESTI, D. P., MASRURI, M., SABARUDIN, A. & SUMITRO, S. B. 2020. Green synthesis and characterization of copper nanoparticles using Piper retrofractum Vahl extract as bioreductor and capping agent. *Heliyon,* 6**,** e04636.
10. SHARMA, M., SHARMA, A. & MAJUMDER, S. 2020. Synthesis, microbial susceptibility and anti-cancerous properties of copper oxide nanoparticles-review. *Nano Express,* 1**,** 012003.
11. SUOOD, A. M., SALEH, M. K. & THALIJ, K. M. Synthesis of Copper Nanoparticles Using Aspergillus Niger and Their Efficacy Against Pathogenic Staphylococcus Aureus. IOP Conference Series: Earth and Environmental Science, 2021. IOP Publishing, 012083.
12. NOOR, S., SHAH, Z., JAVED, A., ALI, A., HUSSAIN, S. B., ZAFAR, S., ALI, H. & MUHAMMAD, S. A. 2020b. A fungal based synthesis method for copper nanoparticles with the determination of anticancer, antidiabetic and antibacterial activities. *Journal of Microbiological Methods,* 174**,** 105966.
13. JAIN, N., BHARGAVA, A., MAJUMDAR, S., TARAFDAR, J. & PANWAR, J. 2011. Extracellular biosynthesis and characterization of silver nanoparticles using Aspergillus flavus NJP08: a mechanism perspective. *Nanoscale,* 3**,** 635-641.
14. AFZAL, H., SHAZAD, S., QAMAR, S. & NISA, S. 2013. Morphological identification of Aspergillus species from the soil of Larkana District (Sindh, Pakistan). *Asian J Agric Sci,* 1**,** e17.

15. MCCLENNY, N. 2005. Laboratory detection and identification of Aspergillus species by microscopic observation and culture: the traditional approach. *Medical mycology,* 43**,** 125-128.

16. DASHEN, M., ADO, S. A., AMEH, J., AMAPU, T. & ZAKARI, H. 2013. Screening and improvement of local isolates of Aspergillus niger for citric acid production. *Bayero Journal of Pure and Applied Sciences,* 6**,** 105-111.

17. SCHWARZ, P., BRETAGNE, S., GANTIER, J.-C., GARCIA-HERMOSO, D., LORTHOLARY, O., DROMER, F. & DANNAOUI, E. 2006. Molecular identification of zygomycetes from culture and experimentally infected tissues. *Journal of Clinical Microbiology,* 44**,** 340-349.

18. ZHANG, Y., ZHANG, S., LIU, X., WEN, H. & WANG, M. 2010. A simple method of genomic DNA extraction suitable for analysis of bulk fungal strains. *Letters in applied microbiology,* 51**,** 114-118.

19. EMBONG, Z., HITAM, W. H. W., YEAN, C. Y., RASHID, N. H. A., KAMARUDIN, B., ABIDIN, S. K. Z., OSMAN, S., ZAINUDDIN, Z. F. & RAVICHANDRAN, M. 2008. Specific detection of fungal pathogens by 18S rRNA gene PCR in microbial keratitis. *BMC ophthalmology,* 8**,** 7.

20. FELSENSTEIN, J. 2008. Comparative methods with sampling error and within-species variation: contrasts revisited and revised. *The American Naturalist,* 171**,** 713-725.

21. SAITOU, N. & NEI, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution,* 4**,** 406-425.

22. ABD EL-AZIZ, A. R., AL-OTHMAN, M. R., ALSOHAIBANI, S. A., MAHMOUD, M. A. & RUSHDY, S. 2012. Extracellular biosynthesis and characterization of silver nanoparticles using Aspergillus niger isolated from Saudi Arabia (Strain KSU-12). *Digest J. Nanomat. Biostruct,* 7**,** 1491-1499.

23. SAITAWADEKAR, A. & KAKDE, U. B. 2020. Green Synthesis of Copper Nanoparticles Using Aspergillus Flavus. *J. Crit. Rev,* 7**,** 1083-1090.

24. UMER, A., NAVEED, S., RAMZAN, N. & RAFIQUE, M. S. 2012. Selection of a suitable method for the synthesis of copper nanoparticles. *Nano,* 7**,** 1230005.

25. DAS, P. E., ABU-YOUSEF, I. A., MAJDALAWIEH, A. F., NARASIMHAN, S. & POLTRONIERI, P. 2020. Green synthesis of encapsulated copper nanoparticles using a hydroalcoholic extract of Moringa oleifera leaves and assessment of their antioxidant and antimicrobial activities. *Molecules,* 25**,** 555.

26. SUBBAIYA, R. & SELVAM, M. M. 2015. Green Synthesis of Copper Nanoparticles from Hibicus Rosasinensis and their antimicrobial, antioxidant activities. *RESEARCH JOURNAL OF PHARMACEUTICAL BIOLOGICAL AND CHEMICAL SCIENCES,* 6**,** 1183-1190.

27. RAMYADEVI, J., JEYASUBRAMANIAN, K., MARIKANI, A., RAJAKUMAR, G. & RAHUMAN, A. A. 2012. Synthesis and antimicrobial activity of copper nanoparticles. *Materials letters,* 71**,** 114-116.

28. KAUR, P., THAKUR, R. & CHAUDHURY, A. 2016. Biogenesis of copper nanoparticles using peel extract of Punica granatum and their antimicrobial activity against opportunistic pathogens. *green chemistry letters and reviews,* 9**,** 33-38.

29. RAHMAN, M., ISLAM, M., BISWAS, M. & KHURSHID ALAM, A. 2015. In vitro antioxidant and free radical scavenging activity of different parts of Tabebuia pallida growing in Bangladesh. *BMC research notes,* 8**,** 1-9.

.